https://doi.org/10.33472/AFJBS.3.4.2021.60-69 ISSN: 2663-2187

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper **Open Access**

Overproduction of Soyasaponin I by *Tipuana speciosa* (Benth.) cell culture

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Article Info

Volume 3, Issue 4, October 2021

Received : 02 May 2021

Accepted : 18 September 2021

Published : 05 October 2021

doi: 10.33472/AFJBS.3.4.2021.60-69

Abstract

Plant cell and tissue cultures are promising biotechnological tools for the production of a myriad of bioactive secondary metabolites on demand. Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have been accelerated in the last few years. Soyasaponins have recently gained more attention due to their biological activities and health-promoting functions. There are many reports relating the bioactive benefits of soyasaponins to their chemical structure mainly Soyasaponin I which possess unique pharmacological actions. Our previous study indicated that *Tipuana speciosa* (Benth.) leaves extract showed promising anti-malarial and nephroprotective activities. This study aimed to isolate Soyasaponin I, a major compound in the leaves extract and the use of cell suspension culture as a biotechnological route for high-yield production of this bioactive molecule. The compound was identified by LC-MS/MS analysis and other NMR techniques. To the best of our knowledge, there is no previous studies have been undertaken for the isolation and the production of Soyasaponin I from *T. speciosa* leaves using plant tissue culture technology. Our data showed that the callus and cell aggregate of *T. speciosa* represent an over producer cell lines (220 folds) compared to the whole plant.

Keywords: *Overproduction; Tipuana speciosa, Soysaponin I, Cell culture*

1. Introduction

Plant cell culture hold a great promise for controlled production of several biologically active secondary metabolites on demand. Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have been accelerated in the last few years (Hussain, 2012). For

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obtaining high yields bioactive molecules suitable for commercial 21 exploitation, efforts have been focused on isolating the biosynthetic activities of cultured cells by optimizing the cultural conditions and selecting high-producing strains (DiCosmo and Misawa, 1995). The major advantages of a cell culture system over the conventional cultivation of whole plants are as follows: (A) Secondary metabolites can be produced under controlled conditions regardless the climatic changes. (B) Cultured cells are deprived of microbes and insects. (C) Reducing labor costs and enhancing productivity. (D) Organic substances are easily extractable from cultures.

Saponins are triterpenoid or steroidal glycosides that occur in several plant species (Waller *et al*., 1993; and Popovich, 2009). The sapogenin aglycons are often substituted with a varying number of sugars *via* an ether or ester bond at one or more glycosylation sites. Saponins are usually located in the seed, leaves, stems and roots of plants (Waller *et al*., 1993; and Popovich, 2009). These compounds are generally associated 31 with plant defense but have also been associated with other biological properties such as insecticidal activity and antifungal activity (Waller 1993, Popovich 2009). They have recently gained more 33 attention because of their bioactivities.

There are many reports relating the bioactive benefits of soyasaponins to their structures such as their potential health-promoting functions, enzyme-altering activity, antimutagenic activity, antiviral activity, hepatoprotective effects, interaction with bile acids, cancer chemopreventation and cholesterol lowering properties (Berhow *et al*. 2002, Popovich 2009). The antiviral functions of soysaponins were demonstrated in the herpes simplex virus type 1. Moreover, soyasaponins have been evaluated for their antiviral activity against *in vitro* HIV, demonstrating that Soyasaponin I inhibit HIV-induced cytopathic effects (Berhow *et al*. 2002). Soyasaponins I (Figure 1) have an inhibitory effect on human cytomegalovirus (HCMV), influenza A virus, and human immunodeficiency virus type. Soyasaponin I is effective in preventing colon cancer by affecting cell morphology, cell growth, and cell proliferation enzymes. Also, it have the potential to reduce the growth of cultured human liver cancer. A majority of soyasaponins were found to induce apoptosis cell death (Berhow *et al*., 2002).

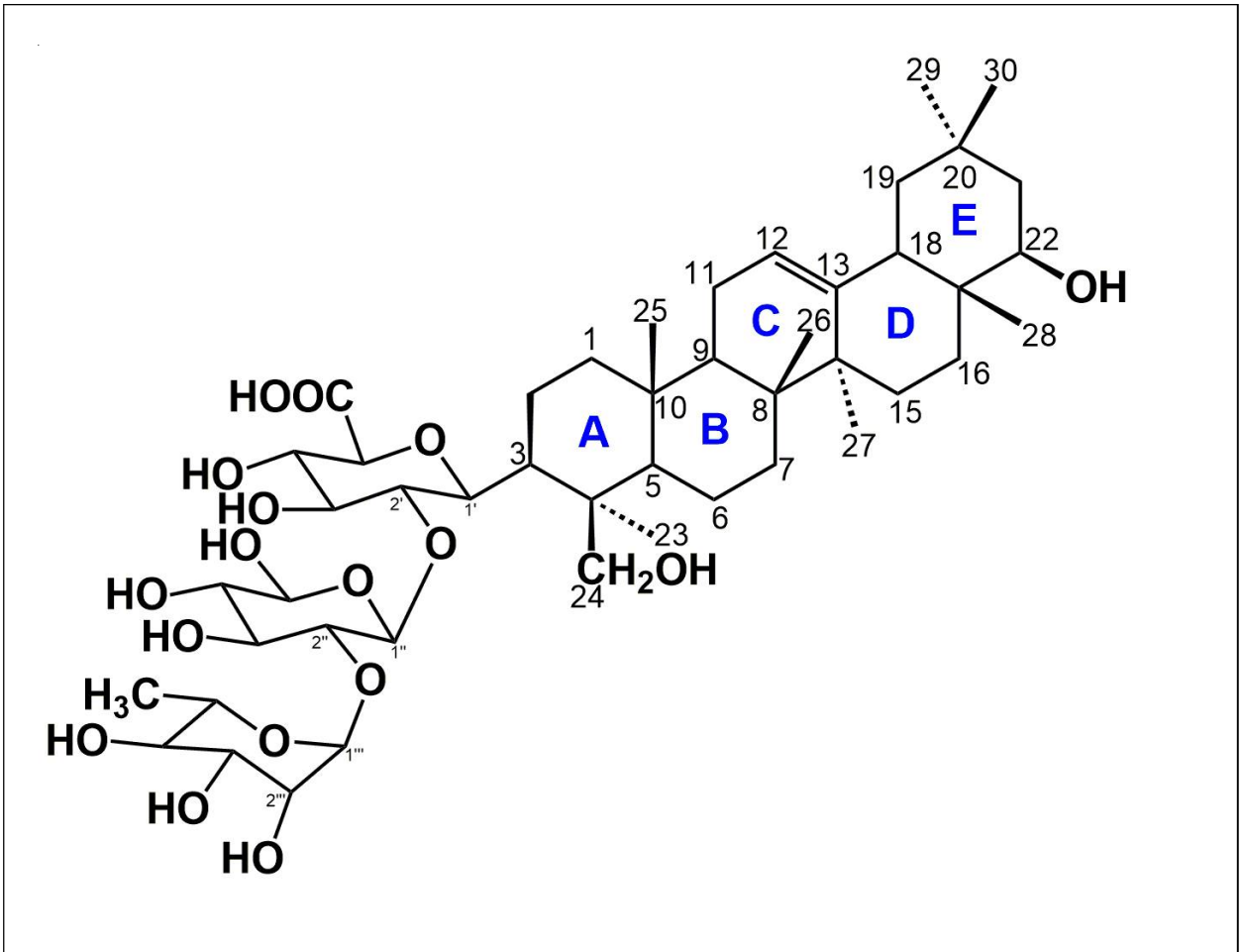

Figure 1: Chemical structure of Soyasaponin I

Some studies have shown that Soyasaponin I possesses free radical scavenging activity comparable to that of α tocopherol inhibiting lipid peroxidation. It has antioxidant effects on rat liver microsome through the inhibition of lipid peroxidation (Berhow *et al*., 2002). Although scientific reports on soyasaponins have been around for at least 80 years, the extraction, purification and quantification still presents many challenges to pharmacognosists. Soyasaponin glycosides are structurally similar; so the purification process is complex, with long sample preparation and extraction times and yielding relatively low amounts of soyasaponins (Berhow *et al*., 2002; and Oleszek and Bialy 2006). We have previously indicated that *Tipuana speciosa* (Benth.) leaves extract showed promising anti-malarial and nephroprotective activities due to their Soyasaponins content mainly Soyasaponin I (Kassem *et al*., 2013).

Here, the work—in hand—is unravelling the overproduction of Soyasaponin I from *Tipuana speciosa* (Benth.) leaves (Figure 2), a widely distributed ornamental tree in Egypt, by tissue culture technology. To the best of our knowledge, no study has been undertaken for the production of Soyasaponin I from *T. speciosa* by tissue culture technology and we report for the first time its isolation from the leaves.

Figure 2. Leaves and legumes of *T. speciosa* Benth

2. Materials and method

2.1. General (solvents and reagents)

Solvents used in for extraction are petroleum ether (40-60 °C), ether, chloroform, ethyl 84 acetate, methanol, n-butanol and ethanol. All solvents were purified according to the methods 85 mentioned by Vogel (1961). All NMR spectra were carried in CD_3OD and DMSO (99.95%, 86 Sigma Aldrich). Solvents used for HPLC analysis were of HPLC grade (Sigma Aldrich Co).

2.2. Plant material

T. speciosa (Benth.) leaves were collected in December 2011 from Azaritta in Alexandria, Egypt. The plant was identified by Prof. Dr. Hosny Abo gazya at Ornamental trees department, Faculty of Agriculture, University of Alexandria, Egypt. A voucher sample was deposited in the Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Egypt.

2.3. Initiation of static and suspension cultures T. speciosa

Using 1.25 % (v / v) hypochlorite solution (Chlorex ®), the leaves were soaked for 15 min to be surface sterilized and then washed three times with sterile water and left to dry on filter paper. MS (Murashige and Skoog-based media, 1962) (Caision Laboratory, USA) was used as the basal tissue culture nutrient. The media pH was adjusted to 6.0 prior to autoclaving at 121 °C and 15 psi for 20 min. Agar (Sigma Chemical Co.) (1% w / v) based MS medium with sucrose (Sigma Chemical Co.) (30 98 g / L) was supplemented with 2, 4-dichlorophenoxyacetic acid (Eastman Kodak Chemical Co., USA) 1.0 ppm (2, 4-D 1). Benzyladenine (Sigma Chemical Co.), 1.0 ppm (BA 1) was used to establish suspension cultures. Liquid cultures were established from static cultures by growing them in the same medium without agar. Static cultures were grown on 5-10 mL of medium in culture vials. Liquid cultures were grown in 50 mL of medium in 100 mL Erlenmeyer flasks. The leaves were cut into 1 cm long pieces. These explants were transferred to MS static medium supplemented with 2, 4-D grown under 16 h photoperiod at 23 °C. The explants stated to form callus at the cut surfaces exposed to the media within approximately one week. The calli were taken over at the end of four weeks. Static culture calli were transferred into the appropriate MS liquid media and grown under photoperiod at 23 °C at 100 rpm using gyrotory shaker (BIOSAN multi-shaker PSU 20, Latvia, Europe) and subcultured every four weeks.

2.4. Fractionation of the total collected suspension culture

Suspension cultures were pooled together in one flask, then the cells were separated out from the media and both were kept aside for fractionation protocol using petroleum ether, n-butanol, chloroform and ethylacetate (all solvents were purchased from Sigma Chemical Co.) as the extracting solvents, respectively. All media extracted fractions did not contain any secondary metabolites as evidenced by the absence of any residue or precipitate after concentration using rotary evaporator. This as well as TLC screening procedures might give an indication that the secondary metabolites formed inside the cells, but not leaked into the culture media. Twenty grams of the dry weight of cultured cells (Callus) were homogenized inside a mortar using neutral sea sand and ethanol (Sigma chemical Co.) as the extracting solvent. The ethanolic extracts were filtered through a cotton piece, then they were freed from the solvent and dissolved in 30 mL of 70% ethanol prior to fractionation protocol using petroleum ether, chloroform, ethyl acetate and *n*-butanol, respectively. The four fractions were concentrated using rotary evaporator and left aside till complete dryness. A white crystalline precipitate was obtained from the *n*-butanolic fraction.

2.5. Isolation of soyasaponin I from the leaves of T. speciosa

Fresh leaves (5.5 kg) of *T. speciosa* Benth. were chopped and exhaustively extracted with 70% ethanol (14 L) by percolation at room temperature. The ethanolic extract was filtered and the solvent was distilled off under reduced pressure at 50 °C. A dark greenish-brown residue (75 g) was dissolved in (1 L) mixture of ethanol and water (7:3) and extracted successively by shaking with petroleum ether (10 × 500 mL), chloroform (8 × 500 mL), ethyl acetate (6 × 500 mL) and *n*-butanol (6 × 500 mL). The solvent, in each case, was evaporated and the corresponding residues were weighed to give 25 g, 2.5 g, 4 g, 33.8 g of Petroleum ether, chloroformic, ethyl acetate and *n*-butanolic fractions, respectively.

A part of the residue of *n*-butanol fraction (10 g) was chromatographed on a column (5 × 70 134 cm) packed with silica (300 g) in methylene chloride. The column was eluted with methylene chloride followed by methylene chloride with increasing amount of methanol. Fractions (100 mL each), were collected. About 62 eluted fractions were collected and similar ones are pooled together according to their TLC behaviors. Two major violet spots and minor faint yellow spots were observed in fractions 46-62. The residue of fractions 46-62 (1.9 g) was washed with a mixture of ethyl acetate and methanol (1:1) then recrystallized from methanol to give white crystals (15 mg) and was designated as compound I.

2.6. Analysis using LC-MS/MS

The resulting precipitate (5 mg) from the cell extract was dissolved in 1 mL of HPLC grade ethanol (Sigma Chemical Co.) and filtered through 0.2 μm micropore syringe filter prior to analysis using LC / MS. The same was performed with 5 mg of the n-butanol fraction of the total crude plant extract.

2.6.1. HPLC conditions

Analyses were performed using a Dionex UltiMate® 3000 HPLC (Germany) composed of a quaternary pump with an on line degasser, a thermostatted column compartment, a photodiode array detector (DAD), an auto sampler, and 1100 Chromeleon® software. The HPLC separation was performed on Eclipse XDB C18 column (50 mm × 2.1 mm, 1.8 μm, Agilent Company, USA). Mobile phase consisted of two solvents, (A) methanol and (B) 0.2% formic acid. Separation of compounds was carried out with gradient elution profile: 0 min, A: B 10:90; 36 min, A: B 100:0; 40 min, A: B 100:0. Chromatography was performed at 30 °C with a flow-rate of 0.2 ml / min. UV traces were measured at 290, 254 and 350 nm and UV spectra (DAD) were recorded between 190 and 900 nm.

2.6.2. Mass spectrometric conditions

The HPLC-MS system consisted of electrospray ionization (ESI) interfaced Bruker Daltonik Esquire-LC Amazon SL ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) and an Dionex UltiMate® 3000 HPLC system equipped with an autosampler and Diode array detector UV spectra (DAD) were recorded between 190 and 900 nm. The ionization parameters were as follows: negative ion mode; capillary voltage 4000 V, end plate voltage –500 V; nebulizing gas of nitrogen at 50.0 p.s.i.; drying gas of 10 l / min nitrogen at 350 °C. Mass analyzer scanned from 70 to 1000 u. The MS–MS spectra were recorded in auto-MS–MS mode. The fragmentation amplitude was set to 1.0 V. MS2 data were acquired in negative mode.

2.7. Identification of compound I by NMR and ESI-MS/MS

The identity of the isolated compound I was further confirmed by using ^{1}H-NMR, HMBC, HMQC, ^{13}C-NMR, LC-MS / MS. The experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpinGmBH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D-NMR spectra were acquired in CD_3OD solvent (99.95%, Sigma Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC and ROESY spectra.

3. Results and discussion

Soyasaponins are amphiphilic molecules, with polar water soluble sugar moieties attached to a nonpolar, water insoluble pentacyclic ring structure. Soyasaponins are categorized according to the individual aglycones (soyasapogenols), and there are two main aglycones, referred to as group A and group B. Group A soyasaponins are bidesmosidic saponins with two glycosylation sites at carbons 3 and 22 on the oleanane ring structure. Group B soyasaponins have one glycosylation site on their aglycones (carbon 3) and are also classified into two groups, based on the conjugation at carbon 22 with a 2, 3-178 dihydro-2, 5-dihydroxy-6-methyl-4-pyrone (DDMP) moiety or non-DDMP conjugated soyasaponins. Non-DDMP conjugated soyasaponins are known as soyasaponins I, II, III, IV and V (Popovich, 2009).

Plant tissue culture is a promising tool for the production of secondary metabolites than the use of the whole plant. Here, in the present work, *T. speciosa* was used for the production of Soyasaponin I by plant tissue culture technology. The fresh leaves of the plant were used as explant for this purpose. The callus was induced on solid culture media using 2, 4-D 1 phytohormone. Callus started to be formed as a light yellowish-green undifferentiated mass after two weeks at the cut surfaces exposed to the solid media (Figure 2).

The HPLC-MS / MS analysis (Figure 3) of the *n*-butnolic fractions of both *T. speciosa* cultured cells (calli) and the dried leaves extract showed major peaks (peak 1 is the one of interest). The retention time, area, area purity and m / z values of peak 1 are summarized in Table 1.

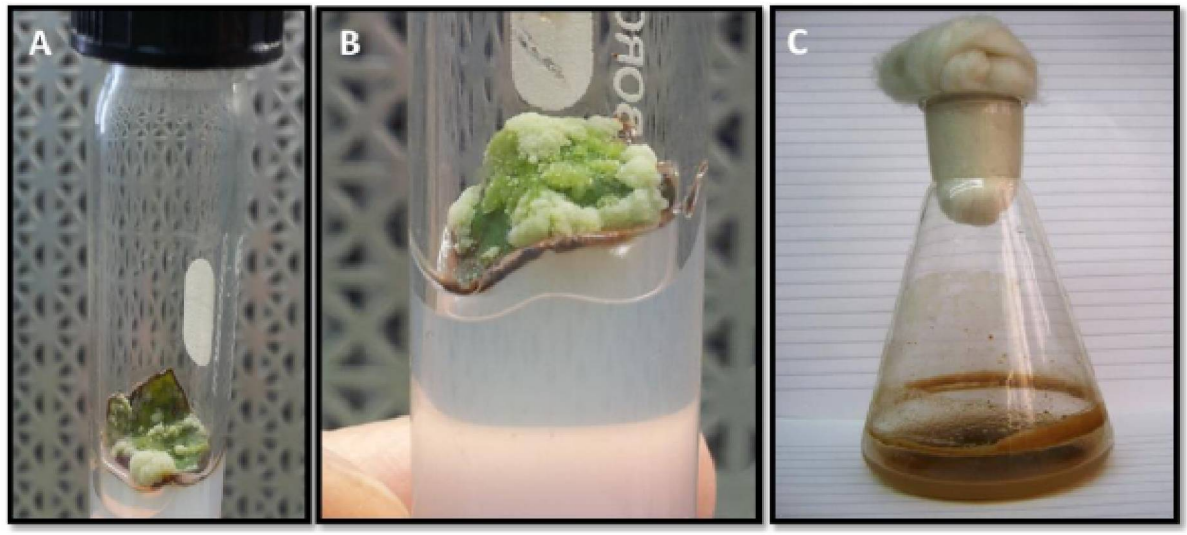

Figure 2. (A) and (B) Callus of *T. speciosa* (Benth.) leaves explant induced on solid media supplemented with 2, 4-D (1 ppm). The callus appeared after four weeks approximately. (C) Suspension culture in liquid MS medium supplemented with BA (1 ppm).

Table 1: Retention time, area purity % and m/z values of the *n*-butnolic fractions major peaks of both *T. speciosa* cultured cells (calli) and the dried leaves extract

Peak#	t_R [min]	Area	Area purity%	Max. *m/z*
1	33	1837040036	100	941.7
1	33.1	815025832	95.4	941.6

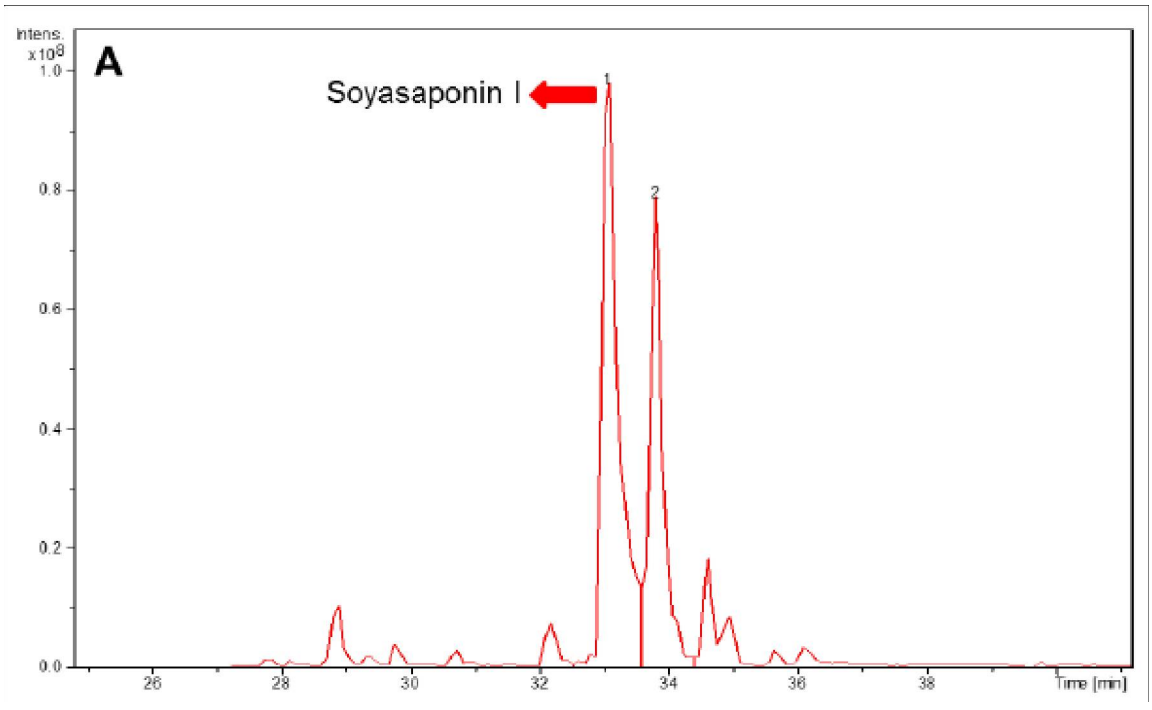

Figure 3. HPLC analysis of (A) the *n*-butanolic fraction of *T. speciosa* Benth cultured cells (B) the *n*-butanolic fraction of *T. speciosa* Benth dried leaves extract. Peak 1 is the peak of interest

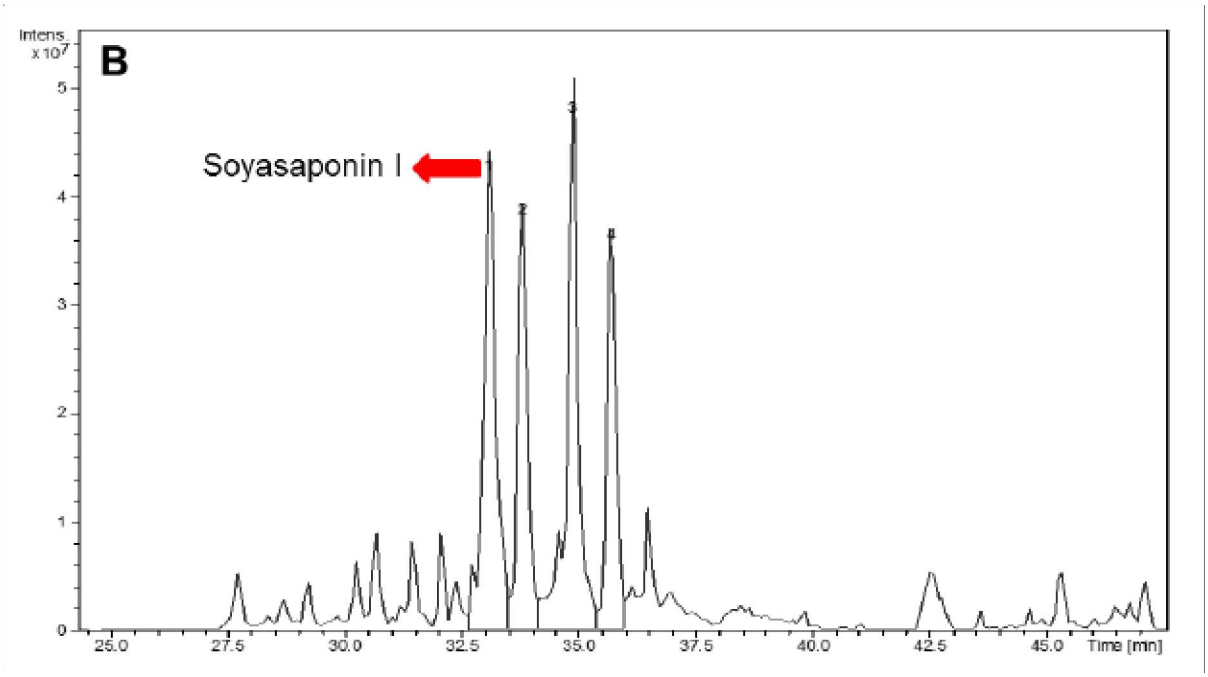

Figure 3: (Cont.)

The negative ion mode ESI-MS spectrum of this peak for the *n*-butanol fraction of *T. speciosa* plant extract (Figure 4) showed a major ion peak at m / z 941.7 assigned for $[M-H]^-$ of compound I and a fragment at *m/z* 795.6 assigned for [M-Rhamnose-H]- which was formed through the loss of rhamnose sugar unit. Another fragment ion at *m/z* 977.2 was assigned for $[M+Na]^+$ in the positive ion mode (Figure 5).

Qualitative chemical reactions and acid hydrolysis of the isolated compound I from the n- butanolic fraction of the leaves extract indicated that is a saponin glycosides (riglycosylated). Also, different spectral data (ESI-MS/ MS, ^{1}H-NMR, ^{13}C-NMR, HMQC and HMBC) suggested that the compound belongs to oleanene type saponin glycoside. The oleanene structure was confirmed by the presence of diagnostic signals in ^{1}H- and ^{13}C-NMR spectra.

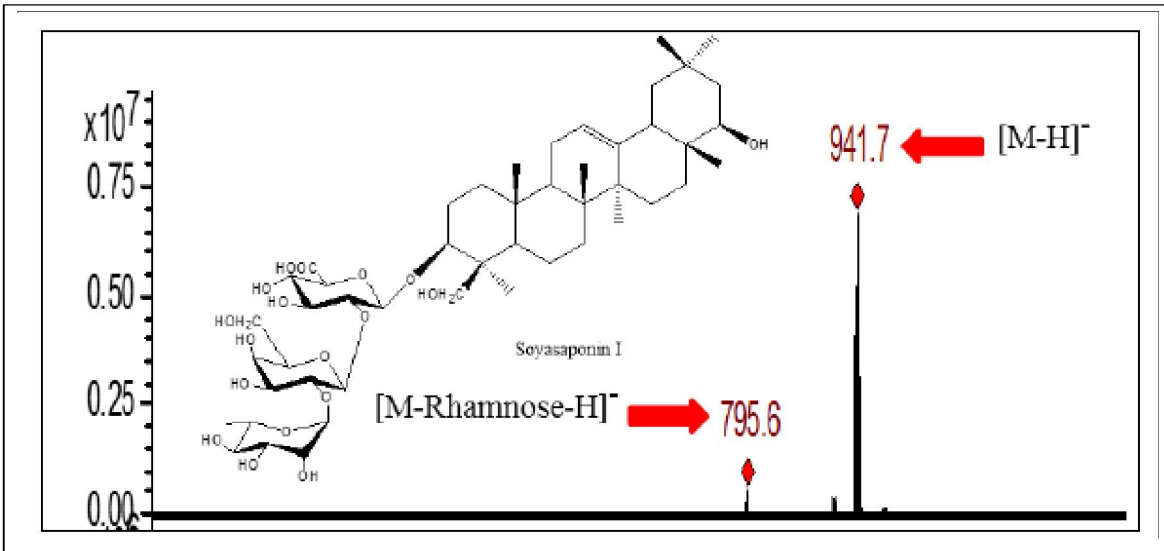

Figure 4: Negative ion mode ESI-MS spectrum of peak 1 in the *n*-butanol fraction of *T. speciosa* callus extract

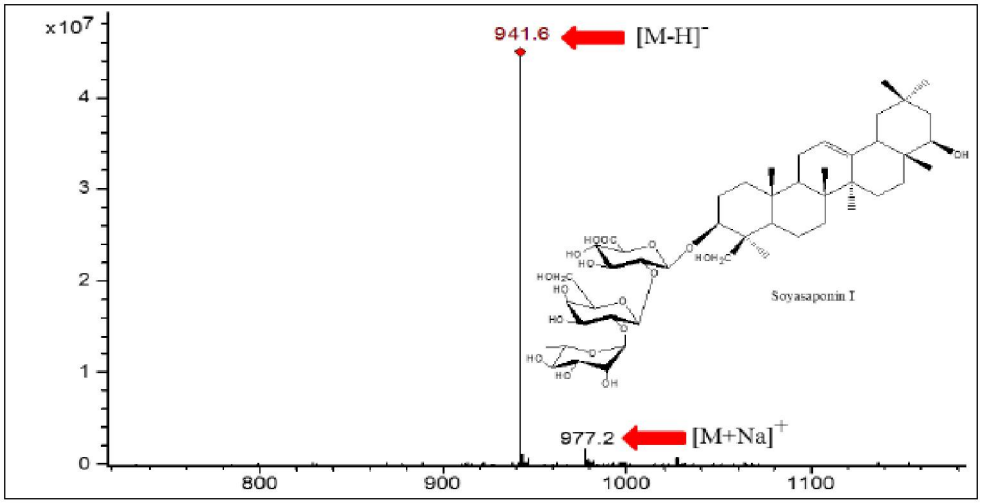

Figure 5: Positive ion mode ESI-MS spectrum of peak 1 in the *n*-butanol fraction of *T. speciosa* callus extract

^{1}H NMR spectrum of the compound (Table 2) revealed the presence of three sugar moieties, by the appearance of three anomeric proton doublets at δ4.48(J = 7.5 Hz), 4.90 (J = 7.9 Hz) and 5.22 (J = 1.2 Hz) (Supplementary Data).

Table 2: ^{1}H and ^{13}C NMR data of the aglycone and the sugar portion of compound I (600 Mz, δ ppm, in CD_3OD)

	Aglycone			Sugar portion	
#	δ_H (J in Hz)	δ C ppm	#	δ_H (J in Hz)	δ C ppm
1	39.6	1.67, 1.07, m		β-D-GlcA (at C-3)	
2	26.7	2.20, 1.85, m	1'	105.4	4.48, d (7.5)
3	92.2	3.42, dd (11.5, 4.2)	2'	77.0	3.79, dd (7.5, 9.0)
4	44.8	-	3'	78.3	3.65, dd (9.0, 9.0)
5	57.2	0.97, m	4'	73.9	3.47, dd (9.0, 9.0)
6	19.3	1.66, 1.42, m	5'	76.9	3.63, d (9.0)
7	34.2	1.60,1.44, m	6'	177.1	-
8	40.6	-			
9	48.9	1.60, m		β-D-Gal (at C-2$_{glcA}$)	
10	37.0	-	1"	101.9	4.90, d (7.9)
11	24.6	1.92, (2H) m	2"	78.0	3.67, dd (8.5, 7.9)
12	123.6	5.27, t (3.5)	3"	76.3	3.52, dd (8.5, 2.9)
13	145.0	-	4"	70.8	3.79, dd (2.9, 1.2)
14	43.3	-	5"	76.2	3.57, m
15	26.5	1.80, 1.09, m	6"	62.0	3.76, dd (12.0, 2.5)3.73, dd (12.0, 4.5)
16	29.6	1.78, 1.38, m		α-L-Rha (at C-2$_{gal}$)	
17	38.3	-	1"'	101.6	5.22, d (1.2)
18	46.5	2.08, m	2"'	72.1	3.95, dd (1.2, 3.2)
19	47.7	1.78, 0.99, m	3"'	71.3	3.75, dd (3.2, 9.7)
20	31.2	-	4"'	74.1	3.43, t (9.7)
21	42.0	1.48, 1.36, m	5"'	69.4	4.12, m
22	76.6	3.42, brs	6"'	18.0	1.30, d (6.5)
23	23.3	1.29, s			
24	64.2	4.16, d (11.4)3.24, d (11.4)			
25	16.3	0.93, s			
26	17.4	1.01, s			
27	25.3	1.16, s			
28	20.2	0.87, s			
29	32.4	0.95, s			
30	28.9	1.04, s			

In the HMQC spectrum (Supplementary Data), these anomeric protons correlated to their anomeric carbons at δ 105.4, 101.9 and 101.6, respectively (Table 2). Complete assignments of ^{1}H and ^{13}C NMR signals of the sugar portion were accomplished by HMQC and HMBC (Supplementary Data) experiments which allowed us to unambiguously assign all proton sugar signals and to identify one glucuronopyranosyl unit (δ4.48) (H1'), one β-galactopyranosyl unit (δ4.90) (H-1") and one α-rhamnopyranosyl unit (δ5.22) (H-1"') (Abbas and Zayed, 2005; and Ma *et al*., 2009). In addition, the β-configuration of glucuronic acid, galactose (J = 7.5Hz) and α-rhamnose (J = 1.2 Hz) were confirmed from the coupling constants of their anomeric protons (Mabry *et al*., 1970).

The sugar moiety of glucuronic acid was further confirmed by the appearance a signal resonating at 177.1 in ^{13}C-NMR spectrum. On the other hand, rhamnose was further confirmed from ^{1}H- and ^{13}C-250 NMR spectra which showed an additional methyl doublet at δ1.3 (J = 6.5 Hz, H-6"') and a corresponding 14 methyl signal at δ18 ppm. The glycosidation site on the aglycone of compound I as well as the position of the interglycosidic linkage were determined by HMBC experiment, which showed long-range correlations between the anomeric proton signal at δ4.9 ($H–1_{gal}$) and the carbon resonance at δ 92.2 (C–3) and between the anomeric proton signal at δ 4.48 ($C–1_{glca}$) and the carbon resonance at δ 77.0 ($C–2_{glca}$). Moreover, comparison of ^{13}C-NMR data of sugar moieties of the compound with those reported for sugar moieties of saponins indicated 1 → 2 linkage between sugars (Pouchet and Behnke, 1993). This was further confirmed by the ESI-MS / MS spectrum (Figure 6) showed an ion peak at *m/z* 965 which was assigned to $[M+Na]^+$, corresponding to a molecular formula $C_{48}H_{78}O_{18}$ (942.5). The MS / MS of this ion showed a peak at *m/z* 819 $[M+Na-146]^+$, due to the loss of rhamnose, at *m/z* 657 $[M+Na-146-260\ 162]^+$, corresponding to the loss of galactose and at *m/z* 481 $[M+Na-146-162-176]^+$, due to the loss of a glucuronopyranosyl unit. The physical, chemical properties and the spectral data of compound I were in full agreement with those reported for 3-*O*-[α-L- rhamnopyranosyl (1"'– 2")- β-D-galactopyranosyl-(1"–2')- β-D- glucuronopyranosyl] soyasapogenol B Known as Soyasaponin I (Abbas and Zayed, 2005; and Takahashi *et al*., 2008).

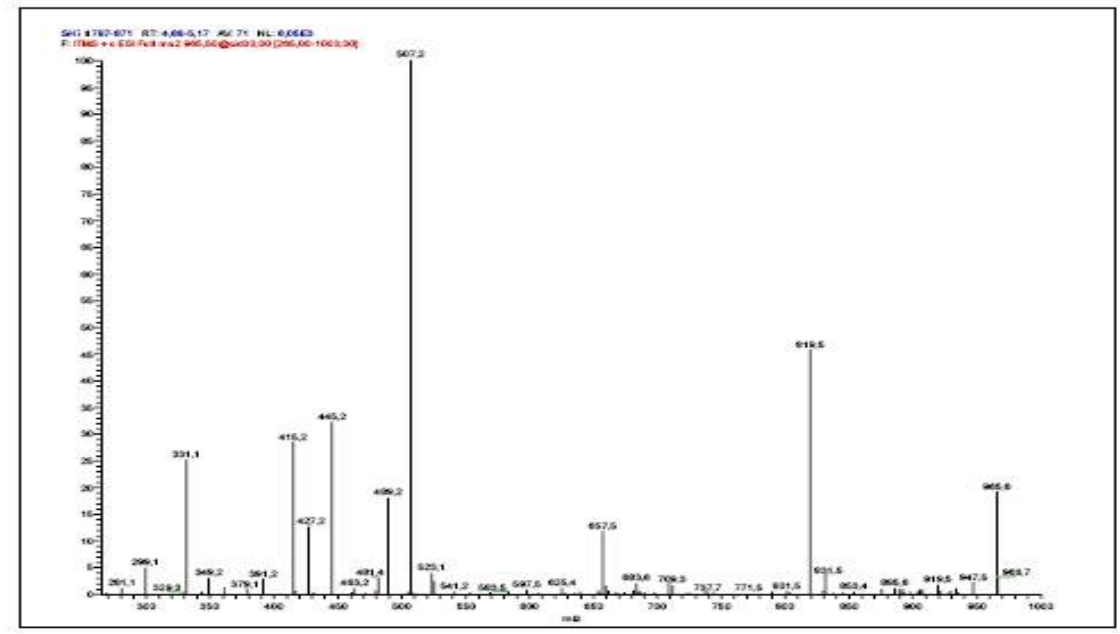

Figure 5: ESI-MS/MS spectrum of compound I

From the previous spectral data, compound I was identified as Soyasaponin I. It is worth mentioning that this is the first report for the isolation of Soyasaponin I from genus *Tipuana*. Moreover, it should be pointed out that 20 gm dry weight of the callus and cell aggregates produced 5 mg of Soyasaponin I (25 % yield), however 5.5 Kg of the whole plant (fresh weight) produced almost 5 mg of the same compound, but with less percentage yield (0.09%). Accordingly, the callus and cell aggregates generated in the present work represent an over producer cell lines (almost 220 folds) compared with the intact plant.

4. Conclusion

Soyasaponin I is a well-known saponin with promising biological activities including antiviral and cytotoxic effects, so we strongly recommend the mass production of this compound by tissue culture technology using

optimized conditions as well as applying some of tissue culture techniques that secure the continuous biosynthetic capacity of the cell lines over extended period of time. Among the proposed tissue culture technological procedures is the immobilization of the over producer cell lines in suitable solid matrices and scaling up the process using a large volumes bioreactor.

Conflicts of interest

No potential conflict of interest was reported by all authors.

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Cite this article as: Fahima F. Kassem, Nagwa S. E. El-Shaer, Fathy K. El-Fiky, Shaimaa M. Sallam and Mohamed A. Abd El-Salam (2021). Overproduction of Soyasaponin I by *Tipuana speciosa* (Benth.) cell culture. *African Journal of Biological Sciences*, 3(4), 60-69. doi: 10.33472 / AFJBS.3.4.2021.60-69.